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14. ABSTRACT

CCCTC-binding factor (CCTF) is a widely expressed 11-zinc finger nuclear protein. CTCF plays an important role in epigenetic regulation by establishing, maintaining epigenetic states in the genome, preventing the spread of DNA methylation, and maintaining methylation-free zones important in gene transcription. CTCF regulates multiple genes involved in cell cycle progression and growth deregulation in both normal and prostate cancer cells. Thus, it serves as a 'master regulator' that when functionally altered has the potential to globally change the behavior of a cell. It is our hypothesis that decreased CTCF expression in prostate cancer modulates the expression of growth promoting and tumor suppressor genes and may be an early permissive change in prostate cancer. Decreased CTCF expression would be associated with changes in local and global methylation especially at DNA binding sites of CTCF target genes in prostate cancer. Specific Aims: (1) Determine if decreased expression of CTCF leads to the deregulation of growth regulatory genes in human prostate epithelial cells and prostate cancer cells. (2) To test if decreased expression of CTCF leads to changes in local and global methylation. (3) Determine CTCF/BORIS expression or function in prostate cancer can predict clinical outcomes. To date, CTCF has not been examined in the context of prostate cancer and no studies have been done studying the effect of CTCF deregulation in normal or nontumorigenic cells. Given its role as a 'master regulator' of multiple genes, this project has the potential to discover a new potential therapeutic target for future studies. Furthermore, CTCF could be used as a marker for prognostic and diagnostic purposes in prostate cancer.

15. SUBJECT TERMS
CCCTC-binding factor (CCTF), DNA methylation, Epigenetics

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INTRODUCTION:

CCCTC-binding factor (CCTF) is a widely expressed 11-zinc finger nuclear protein originally identified as a transcription factor that binds to avian and mammalian MYC promoters (1). CTCF plays an important role in epigenetic regulation by establishing and maintaining epigenetic marks at multiple sites in genome(2). CTCF also prevents spreading of DNA methylation and plays a critical role in maintaining methylation-free zones (3). CTCF has been implicated in control of allele-specific gene expression on imprinted loci and of growing list of promoters involved in cell cycle control, differentiation and apoptosis. Chromosomal band 16q22.1, to which CTCF has been mapped, is frequently found to be deleted in sporadic breast and prostate tumors (4). The genetic loss of CTCF may be involved in dysregulation of number of oncogenes and tumor suppressor genes, including MYC, Rb, PIM1, BRCA1, and IGF2 suggesting that deregulation of CTCF can itself be implicated in cancer development (2). In an analysis of 344 samples of invasive breast carcinoma by differential immunocolocalization, revealed that CTCF abnormally locates in the cytoplasm of 77% of the cases (5). This suggests an important role for the gene in cancer progression and may represent a common feature in cancer cells. The prevailing thought is that reduction of CTCF levels predisposes the cell to epigenetic deregulation at multiple CTCF sites in the genome, inducing progressive silencing of tumor suppressor genes (2). Thus, CTCF could be a 'master regulator' controlling the epigenetic regulation of number of growth and tumor suppressor genes. Previous CTCF work in our laboratory has shown that CTCF declines with senescence using cultured prostate human epithelial cells *in vitro* (6), as well as in aging mouse prostate *in vivo (7).* It is our hypothesis that decreased CTCF expression in prostate cancer modulates the expression of growth promoting and tumor suppressor genes and may be an early permissive change in prostate cancer. Decreased CTCF expression would be associated with changes in local and global methylation especially at DNA binding sites of CTCF target genes in prostate cancer. Additionally, we hypothesize that decreased CTCF expression or function would be a negative prognostic marker for prostate cancer patients.

BODY:

Task 1: Determine if decreased expression of CTCF leads to deregulation of growth regulatory genes in human prostate epithelial cells and prostate cancer cells. (Months 1-12)

a. Establish cell cultures of human prostate cancer (PC-3 and PPC-1) cell lines, HPECs, non-tumorigenic HPV16 E6 and/or E7 prostate cell lines.

> We have established stable cell lines containing inducible CTCF shRNA in pTRIPZ vector in PPC-1, LNCaPs, 293T and non-tumorigenic HPV16 E6 and/or E7 prostate cell lines. We are in process of conducting CTCF knockdown experiments using transient transfection in HPECs.

b. Conduct CTCF shRNA knockdown experiments in above cell cultures.

> We have conducted inducible CTCF shRNA knockdown experiments in the above cell lines. CTCF shRNA was cloned in inducible pTRIPZ lentiviral vector

system (Open Biosystems). Expression of CTCF shRNA is controlled by addition of Doxycycline (2µg/ml) in cell culture media for 3-5 days. Doxycycline treated cells were harvested after 3-5 days and CTCF knockdown was measured using western blots.

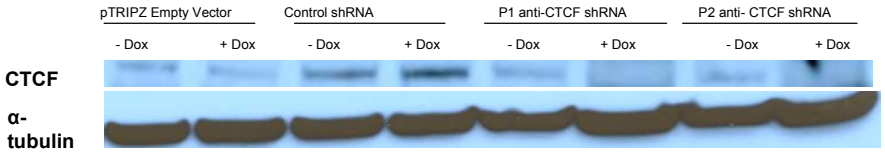

Figure 1. LNCaP inducible CTCF shRNA stable cell line. LNCaP stable cells selected using puramycin (1ug/ml) and shRNA induced using Doxycycline (2ug/ml) for 3 days. CTCF knockdown was seen in cells expressing anti-CTCF shRNA. Controls used were LNCaPs containing pTRIPZ empty vector and cells expressing scrambled shRNA.

Similarly, we have generated PPC-1 stable cell lines expressing inducible anti-CTCF shRNA.

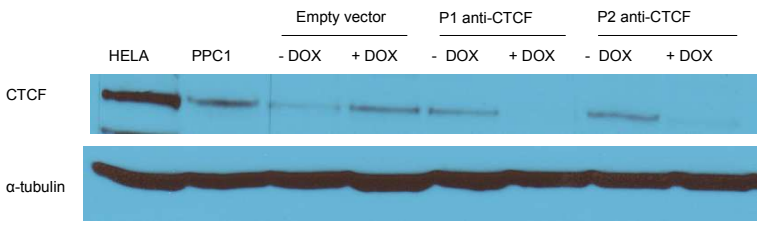

Figure 2. PPC-1 inducible CTCF shRNA stable cell line. PPC-1 stable cells selected using puramycin (1ug/ml) and shRNA induced using Doxycycline (2ug/ml) for 5 days. CTCF knockdown was seen in cells expressing anti-CTCF shRNA. Controls used were PPC-1 containing pTRIPZ empty vector and PPC-1 wild-type and HELA cells.

We have generated stable cell lines expressing inducible anti-CTCF shRNA in 293T cells which express high levels of endogenous CTCF and in non-tumorigenic HPV16 E6 and/or E7 prostate cell lines. We see efficient knockdown of CTCF expression in these cells after induction of CTCF shRNA with doxycycline (data not shown). These stable cell lines generated containing inducible CTCF shRNA would be powerful tools for studying the effects of CTCF knockdown in normal and prostate cancer cells.

c. Conduct quantitative RT-PCR analysis for PTEN, NKX3.1, WT1 and P57 (this has been accomplished for ARF, Rb genes) and western blot analysis.

We are in the process of analyzing target gene expression changes in response to CTCF knockdown in above cell lines using quantitative RT-PCR and western blots.

d. Perform proliferation, cell cycle assays using CTCF shRNA transfected cells.

Proliferation in CTCF knockdown cells was measured as BrdU incorporation into the DNA of cells actively in S phase of the cell cycle. $1.0x10^5$ cells are seeded into 6-well plates and cultured for 3-5 days in growth medium (DMEM +10% FBS) at 37C in 5% CO_2 with or without doxycycline. After this initial culture period, 20 mM BrdU is added to cell-culture medium, 30 min prior to trypsinization and fixed in cold 95% ethanol. These cells are then rehydrated and stained for BrdU and cell cycle phases analyzed using flow cytometry.

In LNCaP cells with CTCF knockdown there was significant decrease in percentage of cells in S phase indicating a decrease in cell proliferation in response to CTCF ablation in these cells. Percentage BRDU incorporation was also significantly reduced in P1 anti-CTCF shRNA expressing cells indicating that CTCF ablation results in decrease in LNCaP cell proliferation.

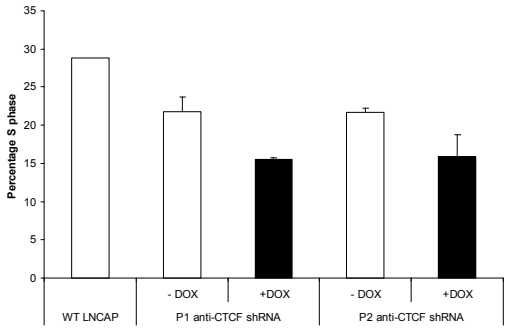

Figure 3. In LNCaP cells, CTCF knockdown with shRNA resulted in decrease in percentage of S-phase cells. P1 anti-CTCF shRNA showed a more marked decrease in cell proliferation (P<0.05). Experiments were done in triplicate.

In PPC-1 cells with CTCF knockdown there was significant decrease in percentage of cells in S phase indicating a decrease in cell proliferation in response to CTCF knockdown in these cells. Percentage BRDU incorporation was also significantly reduced in P1 anti-CTCF shRNA expressing cells indicating that CTCF ablation results in decrease in PPC-1 cell proliferation.

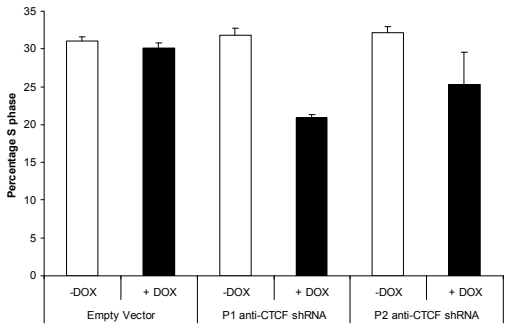

Figure 4. In PPC-1 cells, CTCF knockdown with shRNA resulted in decrease in percentage of S-phase cells. P1 anti-CTCF shRNA showed a more marked decrease in cell proliferation (P<0.05). Experiments were done in triplicate.

We analyzed effects of CTCF knockdown in 293T cells which express high levels of endogenous CTCF expression. We made 293T stable cell line expressing anti-CTCF shRNA and scrambled control shRNA. In 293T cells with CTCF knockdown there was

significant decrease in percentage of cells in S phase indicating a decrease in cell proliferation in response to CTCF knockdown in these cells. Percentage BRDU incorporation was also significantly reduced in P1 anti-CTCF shRNA expressing cells indicating that CTCF ablation results in decrease in PPC-1 cell proliferation.

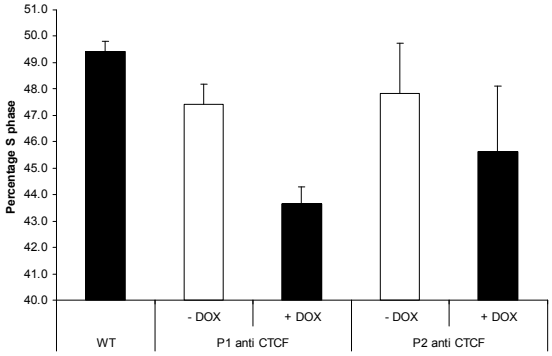

Figure 5. In 293T cells, CTCF knockdown with shRNA resulted in decrease in percentage of S-phase cells. P1 anti-CTCF shRNA showed a more marked decrease in cell proliferation (P<0.05). Experiments were done in triplicate.

e. Generate CTCF shRNA lentivirus.

We have generated shRNA lentivirus containing CTCF specific shRNA, empty pTRIPZ vector and control scrambled shRNA.

f. To test the tumorigenic ability of CTCF shRNA infected non-tumorigenic E6/E7 cells using colony forming assays and tumor xenograft mouse models.

We have made stable cell lines containing anti-CTCF shRNA in non-tumorigenic E6/E7 cells. We are in process of evaluating the effects of CTCF ablation on cell proliferation and cell cycle in these cell lines.

g. Statistically compare the above results.

We find the in all three cell lines (293T, LNCaP and PPC-1) there is significant decrease in cell cycle proliferation and percentage of cells in S-phase in response to CTCF protein knockdown especially in P1 anti-CTCF pTRIPZ construct ($P< 0.05$). This is surprising results as previously it has been reported that CTCF knockdown results in increase in cell proliferation (8). However, in a mice model of CTCF knockout, thymus cells from these mice exhibit growth arrest and decrease in cell proliferation (9). We are in process of evaluating the effects of CTCF knockdown in normal prostate epithelial cells and in non-tumorigenic E6/E7 cells.

Task 2. To determine if decreased CTCF expression leads to changes in local and global methylation. (Months 4-18).

a. Infect HPECs, E6/E7 and prostate cancer cells with CTCF shRNA.
We have done this experiments by generating CTCF shRNA containing stable cell lines in prostate cancer cells (LNCaP, PPC-1), non-tumorigenic E6/E7 cells.

We are conducting transient transfection experiments in HPECs as reported in task1.

b. Determine whether decreased CTCF leads to changes in global methylation using methyl-acceptor assay.

We are in process of conducting these experiments (Months 12-18).

c. Perform methylation mapping and quantitative methylation-sensitive PCR assay for specific changes in methylation in CTCF target genes.

We are in process of conducting these experiments (Months 12-18).

d. Statistically compare the above results.

Once we conduct the above experiments we would be able to statistically analyze the experimental data (Months 12-18).

Task 3. Determine if CTCF expression or function in prostate cancer can predict clinical outcomes. (Months 8-24).

We are in process of acquiring tissue microarrays from John Hopkins University and are planning to utilize the Vectra™ (Caliper Life Sciences, Inc, Hopkinton, MA) instrument for extracting proteomic and morphometric information from tissue microarrays. This system accurately measures protein expressions in distinct tissue regions of interest. We would conduct these experiments between month 12-24 timepoint.

a. Perform microdissection of epithelial and stromal components from men with prostate cancer and extract RNA.

b. Determine CTCF/BORIS expression by quantitative RT-PCR in above samples.

c. Protein expression in tissue microarrays will be determined by immunofluorescence using AQUA^{TM} . Cytoplasmic and nuclear staining will be quantitated.

d. Statistical analysis of CTCF/BORIS expression in different compartments with pathologic and clinical prostate cancer outcomes.

KEY RESEARCH ACCOMPLISHMENTS:

- We have established stable cell lines containing inducible CTCF shRNA in PPC-1, LNCaP, non-tumorigenic HPV16 E6 and E7 prostate cell lines and in 293T cell lines.

- We show that we get effective silencing of CTCF protein in these cell lines expressing CTCF shRNA after inducing the shRNA expression with doxycycline for 3-5 days.

- In prostate cancer cells (LNCaP, PPC-1) and in 293T cells, CTCF knockdown resulted in decrease in percentage of cells in S-phase and decrease of BRDU incorporation. Overall, CTCF knockdown in prostate cancer cells resulted decrease in cell growth response.

REPORTABLE OUTCOMES:

- We have developed PPC-1, LNCaP prostate cancer and non-tumorigenic HPV16 E6 and E7 stable cell lines containing inducible CTCF shRNA whose expression can be controlled by addition of doxycycline to cell culture media.

Manuscripts:
Bhusari, S., Yang, B., Kueck, J., Huang, W. and Jarrard, D. F. (2011), Insulin-like growth factor-2 (*IGF2*) loss of imprinting marks a field defect within human prostates containing cancer. The Prostate. doi: 10.1002/pros.21379

Abstracts:
Bhusari S and David Jarrard. *Epigenetic Alterations Associated with CCCTC-binding factor Deregulation in Prostate Cancer.* "Innovative Minds in Prostate Cancer Today - IMPaCT" conference March 9-12, **2011**, Orlando, Florida.

CONCLUSION:

We have shown that CTCF knockdown in prostate cancer cells (LNCaP, PPC-1) causes decrease in cell cycle as indicated by decrease in percentage of S-phase cells and BrDU incorporation in proliferating cells. These results are in contrast to known role of CTCF as a tumor suppressor gene where CTCF knockdown would lead to increase in cell proliferation (8). There is also a published report of CTCF knockout mice where thymus cells isolated from these mice exhibit cell cycle arrest (9). Our ongoing experiments of CTCF knockdown in normal HPECs, non-tumorigenic HPV16 E6 and E7 cells would help us better understand the role of CTCF in cell cycle progression. Our future experiments would examine local and global methylation changes in response to CTCF ablation in prostate cells. Finally, we would examine the CTCF expression using tissue microarrays and correlate CTCF expression with various clinical parameters.

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